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14. ABSTRACT

In normal cells, proliferation and differentiation are regulated and coordinated in a way that activation of differentiation in normal cells is typically associated with cessation of proliferation. However, the balance between the two is usually disrupted in tumorigenesis, ultimately leading to a complex cellular phenotype typical of cancer cells. In attempts to identify the differentiation-inducing factors in conditioned medium, conditioned medium was fractioned and applied to breast cancer cell, MCF7, cultured in Matrigel. Unexpectedly, a cytotoxic activity was observed on MCF7 cells treated with the fraction of molecular weight between 10-50kDa after seven days. In the past year, I first confirmed the cytotoxicity on various breast cancer cell lines with different genetic backgrounds, including MD-AMB-468 and SKBR3. From mass spectrometric analysis on this 10-50kDa fraction of the conditioned medium, six factors such as interleukins (ILs), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and other cytokines, which are known to be involved in cell proliferation and death, have been selected as proteins of interests and their cytotoxic activities were confirmed by immunodepletion. Chinese hamster ovary (CHO) cells overexpressing each of the six factors have been established for future reconstitution experiments and animal studies.

15. SUBJECT TERMS
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Introduction

In the United States, breast cancer is the most common cancer and second leading cause of cancer mortality in women[1-2]. Our lab is interested in studying the proliferation and differentiation of mammary epithelial cells (MEC) and developing therapies for breast cancer. In normal cells, proliferation and differentiation are regulated and coordinated in a way that activation of differentiation in normal cells is typically associated with cessation of proliferation. However, the balance between the two is usually disrupted in tumorigenesis, ultimately leading to a complex cellular phenotype typical of cancer cells[3-5]. Based on this premise, differentiation-inducing therapies focus on suppressing erratic proliferation by reactivating differentiation in cancer cells. Differentiation therapy is one of the tumor dormancy therapies besides apoptosis-inducing, anti-angiogenic and anti-metastasis therapies as proposed by Uhr[6]. At present, the most successful differentiation-inducing therapy is the application of all-trans-retinoic-acid (ATRA) to acute promyelocytic leukemia[7-9]. ATRA has multiple cellular targets and administration of ATRA induces terminal differentiation of arrested myeloid cells[10-12]. Nevertheless, utilization of tumor dormancy therapy in solid tumors is underdeveloped, despite many solid tumors have been proved to be induced to differentiate *in vitro* by differentiation-inducers which either function in cell-specific or non-specific manner[13].

However, most of these studies on solid tumors were done in two-dimension (2D) culture, which has its intrinsic limitations as over-simplifying *in vivo* microenviroment. *In vitro* 3D culture system utilizing Matrigel is a valuable technique that reproduces *in vivo* microenvironment and has been widely used in studying epithelium development *in vitro*[14-16]. In previous studies, we showed that BRCA1-depleted MECs failed to differentiate in 3D culture and exhibited numerous physiological features of breast cancer cells cultured in Matrigel, including the formation of multilobular aggregates with completely filled lumen[17]. We further demonstrated that formation of acinus structure can be rescued by culturing the cells with conditioned medium collected from differentiating MECs. This observation on conditioned medium suggested that certain factor(s) secreted by MECs might serve in a paracrine/autocrine fashion to suppress cell proliferation but induce cell differentiation, whereas BRCA1-depleted cells lack such activity. In attempts to identify the differentiation-inducing factors in conditioned medium, we fractioned the conditioned medium according to the molecular weight and applied each fraction to breast cancer cell, MCF7, cultured in eight-well chamber slide coated with Matrigel. We observed a cytotoxic activity in the cells treated with the fraction of molecular weight between 10-50kDa and the number of viable cells cultured with this fraction dropped to undetectable after seven days in 3D culture. On the contrary, no effect was observed on normal MECs treated with the same fraction, suggesting this fraction acts only on differentiation-defective breast cancer cells. Identification and characterization of such natural factors that specifically suppress proliferation and induce cell death of breast cancer cells will potentially provide a novel tumor dormancy therapy for treating breast cancer.

Body

RESEARCH DESIGN AND DATA

In our preliminary results, we have demonstrated that 10-50kDa fraction of the conditioned medium collected from MCF10A cells growing in Matrigel has a cytotoxic activity on MCF7 cells also growing in Matrigel. MCF10A cells themselves are not affected under the same treatment. In addition, the highest cytotoxicity is observed on the conditioned medium specifically collected on day 3 and 4 after seeding MCF10A cells in Matrigel. This finding is consistent with the time course of normal acinus formation as on day 3 and 4, MCF10A cells start to differentiate into two distinct populations: a well-polarized outer layer and a poorly-polarized inner layer which eventually die to form the hollow lumen[4]. Before go into the details of identifying cytotoxic factors in the 10-50kDa fraction, I started with repeating the cell killing assay as described for MCF7 cells on other breast cancer cell lines.

1. Test of the cytotoxic activity of the fractionated conditioned medium harvested from differentiating MCF10A on breast cancer cell lines with different genetic backgrounds.

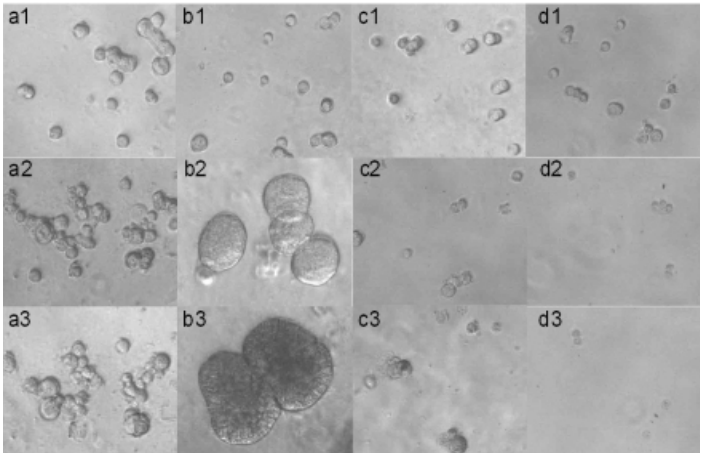
Our preliminary data were based on MCF7 cells which were derived from a pleural effusion containing

metastatic tumor cells from a human mammary adenocarcinoma[18]. MCF7 cells retain several characteristics of differentiated MECs including ability to process 17β-estradiol (E2) via cytoplasmic estrogen receptors (ERs)[19-20]. Basal level of BRCA1 is high in MCF7 partially due to retardation of protein degradation[20]. To exclude the possibility of cell type specificity, I tested the cytotoxic activity of the fractionated medium on three other breast cancer cell lines including SKBR3, HCC1937, MDA-MB-231 and MDA-MB-468, following the same experiment procedures as described for MCF7 cells. Characteristics of these cell lines in terms of disease, derived origin, malignancy and genetic backgrounds are listed below in Table 1[21-27]. As shown in Figure 1 and 2, 10-50kDa fraction of conditioned medium had similar cytotoxic effect on SKBR3 and MDA-MB468 compared to MCF7. MDA-MB231, on the other side, failed to respond to this treatment which may be due to the malignancy of the origin of this cell line (Figure 3). HCC1937 cells were also tested but failed to grow in Matrigel (data not shown). Nevertheless, the cytotoxicity of 10-50kDa fraction of conditioned medium has been confirmed in three breast cancer cell lines with different genetic backgrounds. However, MCF7 was still used as a prototype in most of our later assays.

Table 1. Characteristics of different breast cancer cell lines in terms of origin, malignancy and genetic backgrounds.

Cell line	Disease	Origin	Malignancy	p53	Her2	BRCA1	ER
MCF7	Adenocarcinoma	pleural effusion	metastasis	+	+	+	+
SKBR3	Adenocarcinoma	pleural effusion	metastasis	MU*	+++	–	–
HCC1937	Ductal carcinoma	solid tumor	stage IIB, grade 3	–	–	MU	–
MDA-MB-231	Adenocarcinoma	pleural effusion	metastasis	MU	+	+	–
MDA-MB-468	Adenocarcinoma	pleural effusion	metastasis	MU	–	+	–

* MU, mutated



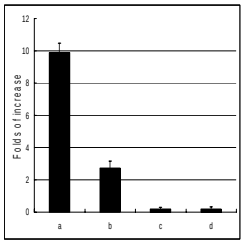
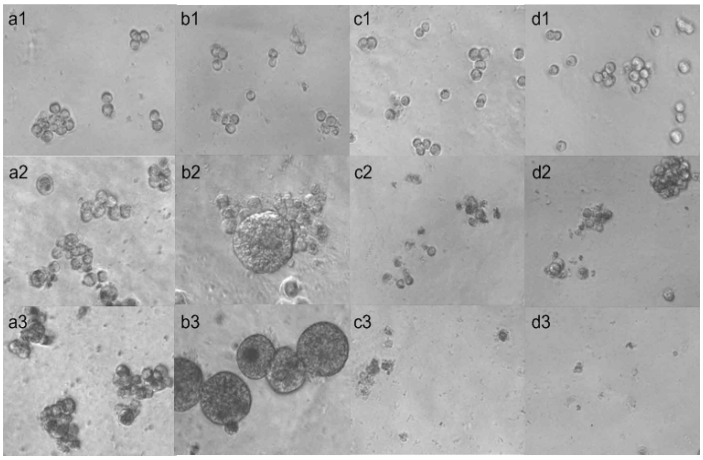


Figure1. Fractionated conditioned medium from differentiating MCF10A suppress proliferation and induce cell death in SKBR3. A) SKBR3 were fed every 12 hours with different fractions of conditioned medium. B) Viable cell numbers were calculated in term of fold of increase. a, no CDMD; b, total CDMD; c, total supernatant; d, 10-50kDa; e, <10kDa.



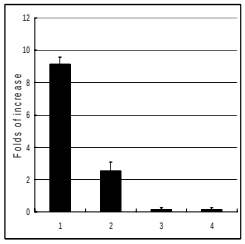
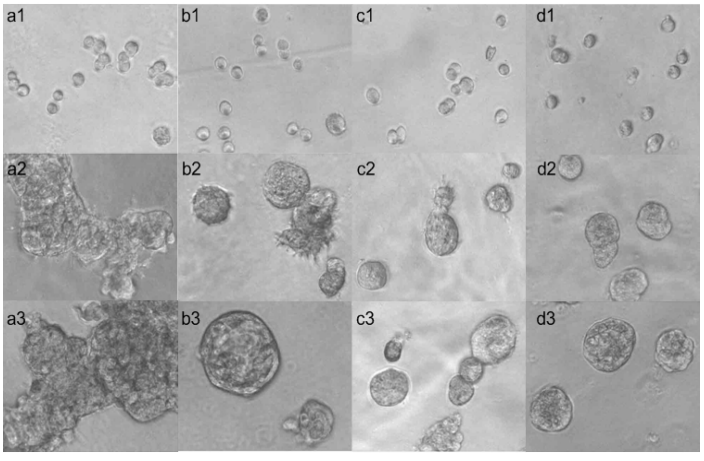


Figure2. Fractionated conditioned medium from differentiating MCF10A suppress proliferation and induce cell death in MDA-MB468. A) MDA-MB468 were fed every 12 hours with different fractions of conditioned medium. B) Viable cell numbers were calculated in term of fold of increase. a, no CDMD; b, total CDMD; c, total supernatant; d, 10-50kDa; e, <10kDa.



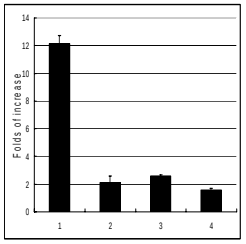


Figure3. Fractionated conditioned medium from differentiating MCF10A suppress proliferation and induce cell death in MDA-MB231. A)MDA-MB231 were fed every 12 hours with different fractions of conditioned medium. B) Viable cell numbers were calculated in term of fold of increase. a, no CDMD; b, total CDMD; c, total supernatant; d, 10-50kDa; e, <10kDa.

2. Identify the secreted factors that have cytotoxic activity on breast cancer cells.

2a) Identification the differentially secreted factors using shotgun proteomics mass spectrometry (MS). As conditioned medium collected from MCF10A cells induces cell death of different breast cancer cell lines growing in Matrigel, it is likely that factors secreted from MCF10A but not breast cancer cells have killing activity. To identify the differentially secreted proteins, I collected the conditioned media from both MCF10A and MCF7 cells cultured in Matrigel every 12 hours for a week. Fractionation was performed as described for cell killing assays and proteins in 10-50kDa fractions were precipitated by trichloric acid. Then, the protein pellets were redissolved and separated on SDS-PAGE by molecular weight. The gel was sliced every 2mm and proteins in each slice were digested with trypsin. After digestion, tryptic peptides were subjected to mass spectrometric analysis using two-dimensional liquid chromatography (strong cation exchange (SCX) as 1st dimension, reverse phase liquid chromatography (RPLC) as 2nd dimension) on-line interfaced with a quadrupole-orthogonal time-of-flight tandem mass spectrometer (QSTAR XL)[28] at UCI core facilities directed by Professor Lan Huang. The acquired spectra were submitted for automated database searching using both Mascot (http://www.matrixsciences.com) and Protein Prospector (http://prospector.ucsf.edu). Thousands of proteins are identified from the conditioned media, most of which are membrane proteins. I compared the two profiles obtained for MCF10A and MCF7 cells and identified factors that present in the medium collected from MCF10A cells but not MCF7 cells, i.e., proteins that are differentially secreted by MCF10A cells. Factors such as interleukins(IL), bone morphogenetic proteins(BMP), fibroblast growth factors(FGF), and other cytokines, are known to be involved in regulating cell proliferation and inducing cell death. We picked up six factors including BMP10, FGF11, antithrombin III (ATIII), IL1F7, IL17E and vitamin D binding protein (VBP) have been chosen as proteins of interest and subjected to further validation in the following steps.

2b) Confirmation of secreted factors by immunodepletion. Based on the cell viability curve over a week (data not shown), media collected on day 3 and 4 showed the highest cytotoxicity. I pooled media collected on day 3 and 4 for fractionation and depletion. To immunodeplete the candidate protein, pooled media were incubated with antibody against the target protein or control rabbit-against-mouse antibody for half an hour in cold room. Protein A/G agarose beads were then added to pull down the antibody-antigen complex. I performed Immunodepletion of all six factors using commercially available antibodies. To confirm the efficacy of immunodepletion, control and target protein-depleted media, as well as the immunoprecipitates, were subjected to Western blot (Figure 4). If the target protein still presented in the supernatant (ie, the media), one more cycle of depletion was performed. The depleted media was then saved for cell killing assay on MCF7 cells growing in Matrigel. Reduction of the cell killing activity after immunodepletion indicates that this depleted factor contributes to the cytotoxicity of the 10-50kDa fraction. As shown in Figure 4, depletion of each individual factor reduces the cytotoxic activity of the conditioned medium to different extents. IL17E (IL25) appears to have a relative high activity while FGF11 and BMP10 have the lowest activities which will not be included in later experiments.

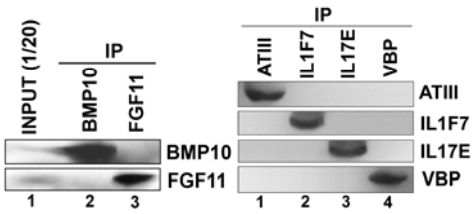


Figure4. Immunoprecipitation of BMP10, FGF11, ATIII, IL1F7, IL17E, or VBP from 10-50 kDa fraction of the conditioned medium.

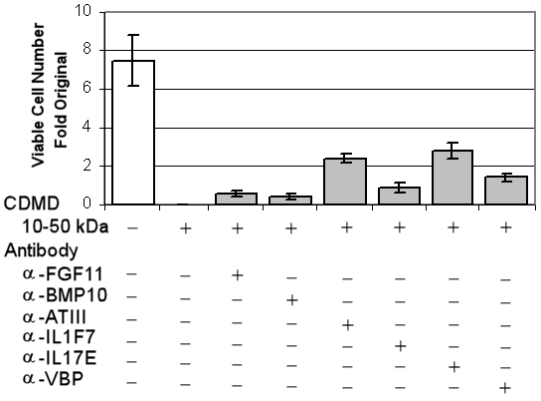


Figure5. Cell viability assay on MCF7 cells after 7 days of growth in 3-D culture with 10-50 kDa fraction of conditioned medium immunodepleted of FGF11, BMP10, ATIII, IL1F7, IL17E, or VBP.

2c) Reconstitution of the depleted medium by adding depleted protein back. To further confirm the requirement of a particular protein for cytotoxic activity, I will reconstitute the conditioned medium by adding the depleted protein back into the medium in which the protein is depleted by either immunodepletion or RNAi. I have already subcloned cDNAs of each candidate protein into pcDNA3.1/Hygro(+) vector for expression in mammalian cells. Human embryonic kidney fibroblast HEK293T cells were first tested for expression. But their

dependence on serum introduced considerable bovine serum albumin contamination to the later purification. Therefore, Chinese hamster ovary (CHO) cells will be used as they can be grown in serum-free medium for better purity. CHO cells will be transfected with each recombinant plasmid and stable cell clones will be selected using antibiotics hygromycin B. Each candidate protein will then be purified from medium using affinity column and further enrichment by fractionation or chromatography may be performed to provide a sufficient amount for reconstitution. If the protein is required for the cytotoxicity, reconstitution of protein should restore the cytotoxic activity of the medium depleted of a particular protein.

Tasks In Progress:

2. Identify the secreted factors that have cytotoxic activity on breast cancer cells.
2c) Reconstitution of the depleted medium by adding depleted protein back.

3. Formulation of a cocktail containing the secreted factors and test of its activity on breast cancer cells in 3D culture system as well as in animals.
3a) Quantification of each secreted factor in conditioned medium to formulate of a cocktail.
3b) *Test of the cocktail in 3D gel.*
3b) Test of the cocktail in animals.

Key Research Accomplishments

- We identified a cytotoxic fraction (10-50kDa) in the conditioned medium collected from MCF10A cells growing in Matrigel which can kill MCF7 cells over a 7-day continuous treatment. This cytotoxicity was confirmed on two other breast cancer cell lines, namely SKBR3 and MDA-MB468, with different genetic backgrounds.
- We found the conditioned media collected specifically on day 3 and 4 have the highest cytotoxicity. We pooled the media collected on these two days and performed mass spectrometry to identify factors within the 10-50kDa fraction.
- By comparing the profiles for MCF10A and MCF7 secreted proteins, we identified over hundreds factors that are differentially secreted by MCF10A cells but not MCF7. Among these, we decided to focus on six factors including FGF11, BMP10, ATIII, IL1F7, IL17E, and VBP.
- We performed immunodepletion of each candidate protein from the conditioned medium and cell killing assay was used to test the cytotoxicity of each immunodepleted medium. IL17E shows the highest cytotoxicity while BMP10 and FGF11 show low cytotoxicity which were then excluded from later experiments.
- The remaining four candidate proteins were expressed in CHO cells individually for purification and reconstitution of immunodepleted conditioned medium.

Reportable Outcomes

NONE

Conclusion

We identified a cytotoxic fraction (10-50kDa) from conditioned medium collected form normal mammary epithelial cells, MCF10A cells, growing in Matrigel. When this fraction was continuously fed onto breast cancer cells MCF7 cells also growing in Matrigel, MCF7 cells were killed in 7 days. This cytotoxicity was confirmed on two other breast cancer cell lines, namely SKBR3 and MDA-MB468, with different genetic backgrounds. The conditioned medium collected on day 3 and 4 have the highest cytotoxicity. From our mass spectrometry analysis on 10-50kDa fraction of the day 3 and 4 pooled conditioned medium, we identified over hundreds factors that are

differentially secreted by MCF10A cells but not MCF7 cells. We selected six genes including FGF11, BMP10, ATIII, IL1F7, IL17E, and VBP as our proteins of interests. By performing immunodepletion and cell killing assay of each candidate genes, we excluded BMP10 and FGF11. IL17E was our major interest as its immunodepleted medium showed the lowest cytotoxicity which means IL17E itself has the highest cytotoxicity on breast cancer cells. We will express the remaining four factors in CHO cells and perform reconstitution and animal experiments.

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Appendices

NONE